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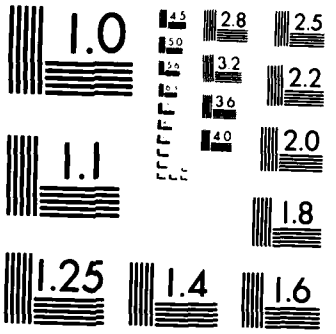

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Our interest is in enzymatically converting type A and B erythrocytes to type O under conditions which render them useful in transfusion therapy. We are currently attempting such conversions of A cells using an α-N-acetylgalactosaminidase (A-zyme) from chicken liver. This enzyme has been purified free of any detectable contaminating proteases, sialidase and other exoglycosidases with the exception of α-galactosidase activity (8-10% of A-zyme) which is believed to be intrinsic and not due to a contaminating enzyme.

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It can remove A antigenicity from the red cell surface and is considerably more effective in doing so than comparable preparations of A-zyme from the earth worm and sheep. Chicken liver A-zyme treated $A_2$ cells (two major A subtypes are $A_1$ and $A_2$) yield haemagglutination titers of 0 when tested with anti-A blood grouping serum, and similarly treated $A_1$ cells yield titers on the order of 1:8 to 1:16. Although the titer of $A_1$ cells can be reduced to 0 with stringent treatment, milder conditions more compatible with the production of transfusable cells are being sought. These include varying the buffer composition and pH and possible supplementary treatment of A antigen-depleted $A_1$ cells with other A-zymes and endo-β-galactosidase from E. freundii. and other microbial sources. Thus far, when the latter is used in conjunction with chicken liver A-zyme, $A_1$ titers are reduced to 1:2 to 1:4. ~~These~~ results are encouraging in that they suggest that A antigenic sites resistant to the action of chicken liver A-zyme are not sterically inaccessible but given the proper enzyme and buffer conditions they too can be removed.

ANNUAL REPORT - 3/1/84 - 2/28/85

ONR Contract N00014-79-0242

# Enzymatic Conversion of Red Cells for Transfusion




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Introduction - Past and Current Accomplishments.

Our overall objective is to effect the conversion of blood types A, B and A B erythrocytes into type O cells under conditions that will make possible their subsequent use in transfusion therapy. We have made significant progress toward the realization of this goal.

Beginning our studies with type B cells because of the availability of alpha-galactosidases we devised treatment conditions which maintain normal cell viability and membrane integrity for both gibbon and human type B erythrocytes. We found that gibbon erythrocytes treated under our conditions with either alpha-galactosidase or alpha-galactosidase Dextran conjugates have normal in vivo survival rates when returned to the original donor. We have thus far performed three in vivo survival studies with human volunteers: The first demonstrated that 1 ml quantities of enzymatically converted human type B erythrocytes could survive normally in the circulation not only of the original B donor but in A and O recipients as well; the second, that a total of 5 mls of converted B cells transfused over a period of four weeks are not immunogenic in A and O recipients as well as the B donor. The third study hich was recently completed involved the transfusion of 20 mls of converted type B cells to an O recipient and the B donor. The converted cells exhibited normal survival rates in vivo and were deemed non-immunogenic since neither an increase in anti B antibody titer nor antibody to the enzyme were detected in either recipient. This last study is the first of a series supported by 6.3 funding provided by the Naval Medical Research and Development Command. The next in vivo survival study will involve the transfusion of one unit of converted B cells to an O recipient.

With regard to the enzymatic conversion of type A erythrocytes to O we are pursuing several approaches which will be described in detail in the next part of this report . After extensive screening of plant, animal and microbiol sources we have isolated and purified a promising alpha- N-acetylgalactosaminidase (A-zyme) from chicken liver. There are three major A subtypes: $A_1$ , Aint, $A_2$ . They are believed to differ in the number of antigenic sites, $A_1$ cells having the most, and in the kind of antigenic structures, $A_1$ cells having some which are not present in the other two. Type $A_2$ comprises approximately 20% to 24% of the A population and Aint on the order of 2% to 4%. The chicken liver A-zyme when used alone will convert type $A_2$ cells to O under conditions favorable for producing cells of transfusable quality. In combination with an endo-beta-galactosidase from Escherichia freundii it will also convert type Aint cells to O. We have not however as yet developed conditions whereby this A-zyme alone or in combination with other enzymes or "activators" will produce a similar conversion of type $A_1$ cells to O.

## Approaches to the Enzymatic Conversion of Type A cell to O.

Our first priority this year was the preparation of sufficient amounts of chicken liver A-zyme to use in determining the conditions for the conversion of all A cell types. We have developed a relatively straightforward procedure for the purification of this enzyme as summarized in Table 1.

Table 1

Purification of α-N-acetalgalactosaminidase from 10Kg chicken liver.

| Fraction | Total Protein | Total Units | Specific Activity | Purification | Recovery |
|---|---|---|---|---|---|
| | mg | μmol/min | μmol/min/mg | fold | % |
| Crude pH4 extract | 300,000 | 6,000 | 0.02 | 1 | 100 |
| 30- 50% $(NH_4)_2So_4$ ppt. | 21,600 | 4,320 | 0.20 | 10 | 72 |
| CM-cellulose | 1,100 | 3,960 | 3.6 | 180 | 66 |
| DEAE-Sephadex | 438 | 3,720 | 8.5 | 425 | 62 |
| Sephadex G-100 | 102 | 2,880 | 28 | 1415 | 48 |
| Chromatofocusing | 47 | 1,980 | 42 | 2100 | 33 |

Briefly, a pH extract is prepared and subjected to a two step ammonium sulfate treatment - the A-zyme precipitating at the 30-50% concentration. Following solubilization of this fraction further purification is attained by ion exchange chromatography. Size separation is then employed using Sephadex G-100 and finally a procedure recently developed by Pharmacia called chromatofocusing. This method is analogous to isoelectric focusing; the proteins being separated according to their isoelectric points except that ion exchangers rather than an electric field produce the separation. Two thousand units of A-zyme are thus obtained from 10 Kilos of chicken liver, a unit being defined as the amount of enzyme hydrolyzing 1 umol of p-nitrophenyl derivative of α-N-Acetylgalactosamine per minute at 37°C under our conditions. The final product has a specific activity of at least 40 representing over a two thousand fold purification, with a recovery of greater than 30%. A-zyme thus purified has been subjected to SDS polyacrylamide gel electrophoresis. Only one band is observed indicating that the enzyme is homogeneous under these conditions.

The properties of chicken liver A-zyme are listed in Table 2.

Table 2

General Properties of α-N-Acetylgalactosaminidase from chicken liver

Glycoprotein

Apparant Mol.wt. ~80,000

Dissociates into subunits at pH 7.5

Apparant subunit Mol.wt. ~40,000

Isoelectric point pH 7.8

Stable at 37° for 18 hrs.

No detectable protease activity

No detectable exoglycosidase activity including sialidase

Exception - 8-10% α-galactosidase activity

No marked activators; inhibited by GalNac - 70%

Gal - 25%; Fuc - 15%; Myoinositol 30%

It appears to be glycoprotein as evidenced by positive periodic acid Schiff staining of the electrophoretic band and binding of the enzyme to con A containing columns. It has an apparent molecular weight. of 80,000 daltons as determined by the procedure of Andrews using Sephadex columns calibrated with proteins of known molecular weight. Its size however is pH dependent since it disassociates into 40,000 dalton subunits at pH 7.5. This disassociation is reversible at lower pHs with complete restoration of activity. Chicken liver A-zyme's isoelectric point of 7.8 is appreciably higher than that of any other A-zyme's which have thus far been reported including the A-zyme from pigeon liver which has an isoelectric point of pH 4.7. We have also observed minor components having slightly different isoelectric points which may in fact be artifacts resulting from the isoelectric procedure. The enzyme exhibits no unusual heat lability and is stable for at least 18 hrs at 37°C. It is not contaminated with any proteases or exoglycosidases including sialidase. The alpha galactosidase activity observed is believed to be intrinsic and has been reported to be present in many alpha-N-acetylgalactosaminidases. Anions and cations do not significantly activate or inhibit the enzyme. As would be expected A-zyme activity is appreciably inhibited in the presence of N-Acetylgalactosamine and to a lesser extent with galactose. There is some inhibition in the presence of fucose, and the polyhidric alcohol, myoinositol, which has been shown to be an inhibitor of some mammalian alpha-galactosidases, inhibits the A-zyme to about the same extent as galactose.

Tabulated in Table 3 are the enzymatic properties of various A-zymes either determined in our laboratory - as for those of chicken, pigeon, sheep liver and earthworm - or taken from the literature as for the last three listed - from pig, human liver and limpet. The pH optimum of chicken liver A-zyme withh a crude salivary glycoprotein preparation is 4, within a range of 3.5 - 4.2. With the p-nitrophenylglycoside derivative it is at the low end of the range at pH 3.7 and shows better than average affinity for this substrate with a Km of 0.67 millimolar and an average rate of reaction with a Vmax of 34 umoles per min. per mg substrate. With the Forssman Antigen which is a ceramide pentasaccharide having a terminal alpha linked N-acetylgalactosomine residue as a substrate the chicken liver enzyme shows the highest pH optimum and a strong Km which somewhat compensates for a weaker than average Vmax. Depending on the substrate, the pH optima of most A-zymes range from 3.5 - 4.7, except for the enzyme from certain species of Clostridia namely perfringens and paraputrificum where it ranges from pH 6-7.

Table 3

Enzymatic properties of α-N-Acetylgalactosaminidases from different sources

| Source | A-active salivary Glycoprotein | p-NP-α-GalNAc | | | Forssman Antigen | | |
|---|---|---|---|---|---|---|---|
| | pH opt. | pH opt. | Km | Vmax | pH opt. | Km | Vmax |
| Chicken liver | 4.0 | 3.7 | 0.67 | 34 | 4.7 | 0.1 | 0.5 |
| Pigeon liver | 3.5 | 3.7 | 0.56 | 31 | ---- | ---- | ---- |
| Sheep liver | ----- | 4.3 | 1.24 | 32 | ---- | ---- | ---- |
| Earthworm | 3.7 | 4.3 | 1.24 | 32 | ---- | ---- | ---- |
| Pig liver | 4.2 | 4.5 | 2.5 | 14.8 | 3.9 | 0.26 | 4.2 |
| Human liver | | 4.3 | 1.3 | 60 | 3.9 | 0.59 | 0 27 |
| Limpet | 4.0 | 3.8 | 0.6 | ---- | 3.8 | 0.036 | ---- |

The general treatment conditions used for enzymatic removal of A antigenicity from the red cell surface are outlined in Table 4.

Table 4

Treatment Conditions

Type A donor red blood cells collected in $CPDA_1$

Packed cells washed 3 x with isotonic saline.

Cells equilibrated 3 x at 5' - 10' intervals with sodium acetate buffer pH 5.7.

Cells incubated at 37° with gentle mixing.

Aliquots removed at various times and tested for changes in A and H activity.

Following incubation cells are washed with PBS pH 7.4 and allowed to come to pH equilibrium ( 30').

Following collection and washing of the packed cells to remove buffy coat they are equilibrated with various pH 5.7 buffers containing either only acetate, acetate-saline or phosphate-citrate-saline. This pH was chosen as a compromise between maintaining cell viability and obtaining reasonable enzyme kinetics. Cells at concentrations ranging between 50-80% are incubated with enzyme usually 50-100 units per 0.25 ml packed cells, in an end-over-end mixer. After testing for changes in A and H antigenicity and determination of the end point of the digestion, the cells are washed with phosphate buffered saline pH 7.4 to remove enzyme and to restore them to a physiological pH.

The effect of such enzymatic treatment upon the removal of A, B and H antigens from all A subtypes as well as O and B cells as determined by serial two-fold hemagglutination titers is shown in Table 5.

Table 5

Hemagglutination titers of cells after treatment with chicken liver α-N-Acetylgalactosaminidase

| Blood Group | Anti A | | Anti A,B | | Mono Anti H | | Anti B | |
|---|---|---|---|---|---|---|---|---|
| | hr | titer | hr | titer | hr | titer | hr | titer |
| $A_1$ | 0 | 1024 | -- | --- | 0 | 4 | -- | --- |
| | 3 | 8-16 | -- | --- | 4 | 128 | -- | --- |
| $A_{int}$ | 0 | 256 | 0 | 1024 | -- | --- | -- | --- |
| | <3 | 0 | 3-5 | 4 | -- | --- | -- | --- |
| $A_2$ | 0 | 256 | 0 | 1024 | -- | --- | -- | --- |
| | 2 | 0 | 4-6 | 0 | -- | --- | -- | --- |
| B | -- | --- | -- | --- | 0 | 64 | 0 | 512 |
| | | | | | 3 | 256 | 3 | 32 |
| O | -- | --- | -- | --- | 0 | 256 | -- | --- |
| | | | | | 3 | 256 | -- | --- |

Under these conditions the enzyme has no effect upon H antigenicity of O cells as measured by a lectin obtained from Ulex which is customarily used to measure H antigen activity and a type 2 chain specific monoclonal anti H kindly provided by Chembiomed Labs. It does reduce B antigenicity from a titer of 1:512 to 1:32 with a concomitant increase in H antigenicity as expected from its inate alpha galactosidase activity. No polyagglutination with A B serum is observed in any of these studies.

Starting titers with both $A_2$ and Aint cells are 1:256 with anti A and 1:1024 with anti A,B under our agglutination conditions. These involve a 10' incubation at room temp. of a 5% saline suspension of cells with antisera, followed by a 1' centrifugation at 3,400 RPM (500RCF), extremely gentle dislodgement of the pellet from the bottom of the tube and both macro and microscopic examination for cell agglutinates. Enzymatically treated type A cells yield zero hemagglutination titers with anti A within two hours. When however, human anti A,B antiserum, a reagent more sensitive than anti A and known to detect weakly reactive minor A cell types is used, no agglutination is observed only after four to six hours. This end point can be shortened if higher enzyme levels are used.

Aint cells operationally defined as producing up to a one plus agglutination with the commercial anti $A_1$, lectin prepared from Dolichos, also yield zero agglutination titers with anti A, but starting titers or 1:1024 with the more sensitive anti A,B are reduced to 4 following enzyme treatment. Neither longer incubation times nor higher enzyme levels significantly reduce this titer.

Similarly, $A_1$ cells have significant amounts of antigenic sites remaining after enzyme treatment, enough to elicit responses with anti A of up to 1:8 or 16. These sites are also resistant to higher enzyme concentrations and longer incubation times. H antigencity increases as would be expected, going from a 4 at zero time to a titer of 1:128 following reaction of the cells with enzyme indicating that chicken liver A-zyme is cleaving only terminal alpha-N-acetylgalactosamine residues from the antigen containing glyco moiety. In an attempt to remove the remaining A antigens from such A1 depleted cells we have reequilibrated them with fresh buffer and reincubated them either with chicken liver enzyme alone or in combination with small quantities of A-zymes isolated in our laboratory from pigeon and sheep livers. No further decrease in titer resulted from any of these treatment.

As part of this approach we are currently studying an A-zyme from the non-pathogenic Clostridium paraputrificum first identified in this microorganism by Dr. Nicholas Paoni then working at the Naval Biosciences Laboratory and made available to us through the efforts of Dr. Jaennine Majda. We have isolated the enzyme, purified it free of contaminating sialidase and proteases activities and reduced its two major exolycosidase contaminants: beta galactosidase and beta N-acetylglucosaminidase from 60% and 24% to 2.9% and 1.6% respectively. Such an enzyme preparation when incubated with red cells still causes them to become polyagglutinable but to a much lesser extent than either Paoni's or our crude enzyme preparations and has allowed us to determine that this A-zyme can remove A antigen from the red cell surface. The next step is to remove the remaining exoglycosidase contaminants and if necessary other impurities in order to produce an enzyme preparation that will not cause cells to become polyagglutinable, and then test it with our A antigen depleted A1 cells to determine if it can attack those A antigenic sites resistant to chicken liver A-zyme.

Protein "activators" have been isolated from mammalian livers and shown to enhance the activity of some exoglycosidases from the same source when used with glycolipid substrates. We have isolated three such fractions from chicken liver which produce similar results with an A antigen containing glycolipid. Treatment of $A_1$ cells with chicken liver A-zyme in the presence of such "activators" does not produce a further reduction in their hemagglutination titer indicating that these substances do not affect A-zyme activity at least with respect to the removal of A antigens from the cell surface.

$A_1$ cells treated with chicken liver A-zyme will yield a zero agglutination titer with anti A when incubated in a high citrate containing buffer also consisting of phosphate, dextrose and sucrose at pH 5.3 and 37°C. Detailed examination of such buffers is ongoing. It appears that high concentrations of citrate at pH 5.3 affect the red cell membrane producing among other things a decrease in the expression of A antigencity and membrane damage thus rendering the cells unfit for transfusion therapy. Attempts are being made to devise a buffer system which will perturb the membrane only enough to reduce A antigenicity without producing irreparable membrane damage.

Another approach being taken involves the treatment of $A_1$ cells with a combination of chicken liver A-zyme and endoglycosidases. The current endo enzyme in use is an endo-beta galactosidase found in several microorganisms. This kind of endoglycosidase cleaves beta galactosidic linkages adjacent to either N-acetylglucosamine or glucose of repeating lactosamine structures although no hydrolysis occurs at adjacent branched polysaccharide structures. It has been shown that this enzyme hydrolyzes the carbohydrate chains expressing ABH and I and little i antigenic activities.

Table 5

Hemagglutination titers of cells after treatment with α-N-Acetylgalactosaminidase and endo-β-galactosidase

| Blood Group | Anti A | | Anti A,B | | Anti I | | A B Serum | | Mono Anti H | | Anti B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | hr | titer | hr | titer | hr | titer | hr | titer | hr | titer | hr | titer |
| $A_1$ | 0 | 1024 | 0 | 2048 | 0 | 16 | 0 | 0 | 0 | 4 | -- | --- |
| | 3 | 2-4 | 3 | 32 | 3 | 0 | 3 | 0 | 3 | 4 | | -- |
| $A_{int}$ | 0 | 256 | 0 | 1024 | -- | --- | 0 | 0 | -- | --- | -- | - |
| | <2 | 0 | 6 | 0 | -- | --- | 6 | 0 | -- | --- | -- | --- |
| B | -- | --- | -- | --- | -- | --- | -- | --- | 0 | 64 | 0 | 512 |
| | -- | --- | -- | --- | -- | --- | -- | --- | 3 | 4 | 3 | 32 |
| O | -- | --- | -- | --- | -- | --- | -- | --- | 0 | 256 | -- | --- |
| | -- | --- | -- | --- | -- | --- | -- | --- | 3 | 16 | -- | --- |

Treatment of $A_1$ cells with endo-beta-galactosidase from Escherichia freundii using from 10 milliunits to 100 milliunits per 250 microliters of cells only produces a decrease in titer from 1024 to 512. In combination with chicken liver A-zyme it produces, as shown in Table 6, an expected decrease in H antigenicity with both O and B cells and no change in anti H titer, which actually reflects a loss for $A_1$ cells and also the previously reported loss of I antigenicity. Treatment of Aint cells with this enzyme plus our A-zyme results in a zero agglutination titer with anti A,B antisera instead of the 1:4 obtained with A-zyme alone. Similar treatment of $A_1$ cells yields titers never greater than 1:2-4 reduced from 1:8-16 obtained when these cells are incubated only with chicken liver A-zyme. As before, treatment of these A antigen depleted $A_1$ cells with the A-zymes from pigeon or sheep liver does not change their titer nor is there any effect with higher chicken liver enzyme concentrations or longer incubation times. It should be mentioned that thus far no polyagglutination is observed with any of these exo and endo-glycosidically treated cells when they are tested with their own or AB serum.

The results obtained by the combined A-zyme and endo-beta-galactosidases treatament of A cells showing a further decrease in hemagglutination titer are encouraging in that they suggest that A antigenic sites resistant to the action of chicken liver A-zyme are not sterically inaccessible to enzyme action but given the proper enzyme and buffer conditions they too can be removed. As further support for this hypothesis recent preliminary results with an endo-beta-galactosidase we are isolating and purifying from Cytophaga keratolytica indicate that much if not all remaining A antigens are susceptible to higher levels of enzyme on the order of ten - twenty times more than previously used. Further purification of this enzyme is underway so that larger amounts can be used to treat $A_1$ cells.

Goals for 1985 - 86

With these results in mind our goals for the forthcoming year will be: To complete the standardizing of the treatment conditions for the conversion of $A_2$ - Aint cells; to perform in vitro membrane and metabolic studies of such enzymatically converted cells - the accomplishment of both these goals will make feasible the transitioning of such converted cell studies to 6.3 funding for the subsequent human studies. Our final goal will be to develop the proper conditions in terms of determining the necessary enzyme or combination of enzymes, buffers, etc. needed for the conversion of $A_1$ cells to O cells of transfusable quality.

Culmulative Publications List.

"The Preparation and Properties of An α-Galactosidase Immobilized on a Soluble Support." J.-V. Kuo and J. Goldstein, Fed. Proc. 38, 418 Abs. #998 (1979).

"Removal of Erythrocyte Surface Antigens by and α-Galactosidase and its Dextran Conjugate." J. Goldstein, J.-Y. Kuo, L. Lenny. Joint Meeting of 18th Cong. Internat'l. Soc.of Haematology & 16th Cong. Internat'l Soc.Blood Transfusion, Montreal, Canada Abs. p298 #1634, 1980

"Nonantigenic Red Blood Cells." J. Goldstein in "Current Concepts of Combat Casualty Resuscitation Symposium". In press.

"Enzymatic Removal of Blood Group B Antigens from Gibbons Erythrocytes". L. Lenny and J. Goldstein. Transfusion, 20 618, 1980.

"Normal Survival of Enzymatically Converted Group B Erythrocytes in O, A and B Recipients." J. Goldstein, G. Siviglia, R. Hurst, L. Lenny and L. Reich. Transfusion 21, 602-603, 1981.

"Enzymatic Conversion of B Erythrocytes to Group O Yields Normal Survival in O, A and B Recipients.," J. Goldstein, G. Siviglia, R. Hurst, L. Lenny and L. Reich. Science 215, 168-170, 1982.

"Liquid Nitrogen Preservation of α-Galactosidase-treated Erythrocytes." L. Lenny, J. Goldstein and A. W. Rowe. Cryobiology, 19, 678-679, 1982.

"Cryopreservation of Enzymatically Converted B→ O Erythrocytes With and Without Metabolic Rejuvenation." L. Lenny, J. Goldstein, and A. W. Rowe Transfusion 22, 420, 1982.

"Enzymatic Conversion of Red Cell ABO Groups for Transfusion." J. Goldstein. In "Recent Advances in Haematology, Immunology and Blood Transfusion. Eds. S. R. Hollan et al. Akademiai Kiado, Budapest. 89-97, 1983.

"An α-Galactosidase Immobilized on a Soluble Polymer." J.-Y. Kuo and J,. Goldstein, Enz. & Micro. Tech. 5, 285-290, 1983.

"Preparation of Transfusable Red Cells by Enzymatic Conversion." J. Goldstein Sixth Red Cell Conference. George J. Brewer, Ed. Allan R. Liss, New York PP. 139-157, 1984.

"Enzymatic Removal of Group A Antigens from the Red Cell Surface." Abstracts of the Eighteenth Congress of the Internation Society of Blood Transfusion, Munich, July 22-27, 1984 p.86.

"Biochemical Manipulation of Blood Groups." J. Goldstein, Clinics in Immumnology and Allergy, J.J. van Rood, R.R.P. de Vreis, Eds. W. B. Saunders. London. Vol. 4, No. 3, October 1984.

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